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Sequence Complexity and Relative Abundance of Vaccinia Virus mRNA's Synthesized In Vivo and In Vitro

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The sequence complexity and relative abundance of vaccinia virus mRNA's, synthesized in vivo and in vitro, have been measured by DNA-RNA hybridization. Up to 42% of [^{3}H]thymidine-labeled virus DNA can be protected from digestion with nuclease S_1, a single-strand-specific nuclease, after annealing to excess polyadenylylated mRNA obtained at 7 h after infection. In contrast, only 26% of vaccinia virus DNA is protected when hybridized to polyadenylylated RNA obtained at 2 h after infection in the presence of an inhibitor of DNA synthesis. That the 94 kilobases transcribed early are a subset of the 152 kilobases present late was suggested by hybridization of DNA with a mixture of early and late RNAs. Some control of transcription is lost when virus purified by procedures that include sonic treatment is used for infection since under these conditions similar proportions of DNA are protected by either excess early or late RNA. Excess RNA, synthesized in vitro by enzymes within purified vaccinia virus particles, hybridized to approximately the same fraction of the DNA as did RNA present at late times in vivo. A second type of transcriptional control was demonstrated by kinetic analysis of the hybridization of polyadenylylated RNA to labeled DNA. With virion DNA used as the probe, a single abundance class for early RNA, two classes differing 11-fold in abundance for late RNA, and two classes differing 43-fold in abundance for in vitro RNA were found. To be able to detect high-abundance RNAs of very low sequence complexity, labeled complementary DNA probes to early, late, and in vitro polyadenylylated RNA were used. Evidence that, at late times, RNAs totaling 9 kilobases of sequence complexity are present 40 to 500 times more frequently than the bulk of the virus-specific RNA was obtained. In contrast, the highest abundance class of RNA present at 2 h after infection corresponded to 7 kilobases present in only a 13-fold molar excess over the majority of virus-specific sequences. RNA synthesized in vitro was found to contain a small amount of sequence information, approximately 2 kilobases, which occurred 150 times more frequently than the majority of viral sequences. Studies using hybridization of viral DNA to labeled complementary DNA probes also suggested that 52 to 59% of the polyadenylylated RNA present at 2 h after infection and 82 to 92% of that at 7 h are virus specific.

Vaccinia virus provides a useful system for studying the mechanism and control of transcription both in vitro and in vivo. The presence within purified virus particles of a DNA-dependent RNA polymerase (24, 34), as well as enzymes that modify the 5′ (44, 45) and 3′ (23, 32) ends of nascent molecules to form characteristic "eucaryotic-type" mRNA in vitro, is a unique feature of poxviruses. In addition, the cytoplasmic site of vaccinia virus replication and transcription facilitates the detection of virus-specific RNA in vivo (21). Evidence for regulation of gene expression during the growth cycle of vaccinia virus was initially obtained from studies of protein synthesis (for a review, see reference 30). Early proteins, made during the first few hours of infection, and late proteins, made exclusively after viral DNA replication, have been identified. Nevertheless, the large size of the genome, approximately 123×10^6 daltons (13), has hindered efforts to characterize vaccinia virus-specific RNA. Competition-hybridization experiments, carried out by Oda and Joklik (36), indicated that RNA made before virus DNA replication contains only half of the vaccinia virus-specific sequences found at late times. Using a similar competition-hybridization analysis, Kates and Beeson (22) reported that RNA made in vitro by vaccinia virus cores represents a subset of the early RNAs made in vivo. Recently,

more quantitative estimations of the proportion of the poxvirus DNA transcribed have been made by Kaverin and co-workers (25) and Paoletti and Grady (39). The major objectives of the present study were to determine the sequence complexity of vaccinia virus-specific RNAs synthesized in vitro and in vivo and to provide information regarding the existence of RNA abundance classes.

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MATERIALS AND METHODS

Infection of cells. HeLa S-3 cells, growing in suspension culture at 37°C, were concentrated to 5×10^6/ml and infected with 15 PFU of vaccinia virus per cell. Virus was purified either with (29) or without sonic treatment. When sonic treatment was omitted, the virus was treated at 37°C for 30 min with 0.25 mg of 2× crystallized trypsin per ml before sedimentation through a 36% sucrose cushion and again before sedimentation in a sucrose gradient. The particle-to-PFU ratio calculated from the optical density at 260 nm (OD_{260}) (1 OD = 1.2×10^{10} particles) and by titration on monolayers of BSC-1 cells was 41 for the preparation of sonically treated virus and 58 for virus purified without sonic treatment. When crude virus stocks (9.3×10^9 PFU/ml) were used for infection, they were treated with trypsin (1.25 mg/ml) for 30 min at 37°C (33). After a 30-min virus-absorption period, the cells were diluted to 5×10^5/ml. In some experiments, cytosine arabinoside (40 μg/ml) or cycloheximide (100 μg/ml) was added at 15 min before virus infection.

Isolation of polyadenylylated RNA. At appropriate times after infection, cells were washed twice with isotonic saline at 4°C and then suspended in hypotonic buffer containing 10 mM Tris-hydrochloride (pH 9.0), 10 mM NaCl, and 1.5 mM $MgCl_2$ for 15 min at 0°C. After Dounce homogenization and centrifugation at $350 \times g$ to remove nuclei, the cytoplasmic fraction was adjusted to 2% Sarkosyl and the RNA was pelleted through cesium chloride to remove DNA and proteins (15). The pelleted RNA was dissolved in buffer containing 0.1 M NaCl and 10 mM Tris-hydrochloride (pH 7.6), and the absence of contaminating DNA was confirmed by electron microscopy. After the addition of 3 volumes of ethanol and storage at −20°C, the RNA was collected by centrifugation at $10,000 \times g$ and suspended in 0.12 M NaCl–2 mM EDTA–10 mM Tris-hydrochloride (pH 7.6)–0.1% sodium dodecyl sulfate (SDS) (buffer A). This material was applied to a polyuridylic acid [poly(U)]-Sepharose column (0.7 by 6 cm) at 24°C and washed with 50 ml of buffer A. The polyadenylylated RNA was eluted from this column with 90% formamide–10 mM Tris-hydrochloride (pH 7.6), incubated at 68°C for 2 min, diluted with 5 volumes of buffer A, and applied to a second poly(U)-Sepharose column. The RNA eluted from the second column was precipitated in 75% ethanol at −20°C, collected by centrifugation, and resuspended in 10 mM Tris-hydrochloride, pH 7.6. The RNA concentration was determined in a Cary recording spectrophotometer, using 1 OD_{260} as the equivalent of 45 μg of RNA per ml. Both early and late viral RNAs labeled with [^{3}H]uridine sedimented heterogeneously in sucrose gradients with a peak between 8 and 12S.

In vitro RNA synthesis. Vaccinia virus, at a concentration of 1 OD_{260} per ml, was used for in vitro RNA synthesis (7). Virus cores were removed by centrifugation, and the RNA was obtained by precipitation with 0.025% cetyltrimethylammonium bromide (43). Approximately 8 μg of polyadenylylated RNA was recovered per OD of virus. The RNA sedimented heterogeneously in sucrose gradients with a peak between 8 and 10S. In some experiments, the RNA was also sedimented through CsCl before poly(U)-Sepharose chromatography.

Purification of labeled vaccinia virus DNA. HeLa cells were infected with crude vaccinia virus at 1 PFU/cell in medium containing 25 μCi of [^{3}H]thymidine (5.5 Ci/mmol) per ml. At 20 h after infection, the cells were collected and the labeled virus was purified (29). The purified virus was suspended in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA–1% Triton X-100 and then sonically treated six times for 15-s intervals at 0°C with the microtip of a Branson Sonifier. After treatment for 3 h at 37°C with protease K (1 mg/ml), the viral DNA was sonically treated as above, extracted twice with phenol, and passed through a column of Sephadex G-50 equilibrated with 50 mM ammonium acetate, pH 6.0. The specific activity of this DNA was 5.4×10^6 cpm/μg. The fragmented DNA sedimented in an alkaline sucrose gradient (0.7 M NaCl–0.3 M NaOH) with a peak at approximately 6S.

DNA-RNA hybridization. Hybridization reactions contained 1.0 M NaCl, 10 mM EDTA, 10 mM Tris-hydrochloride (pH 7.6), 200 μg of yeast RNA per ml, 150 μg of salmon sperm DNA per ml, 70 ng of [^{3}H]thymidine-labeled vaccinia virus DNA per ml, and from 6 to 70 μg of polyadenylylated RNA obtained from vaccinia virus-infected HeLa cells per ml. Polyadenylylated RNA obtained from uninfected HeLa cells was used in control hybridizations. The concentration of the RNA driver was 100 to 1,000 times that of the labeled vaccinia virus DNA. Each hybridization mixture was denatured by boiling for 5 min before the addition of salt, and 100-μl samples were then sealed in capillary tubes and incubated at 68°C, which is 15°C below the T_m of vaccinia virus DNA (R. Parr, personal communication).

To assay for the percentage of ^{3}H-labeled vaccinia virus DNA that formed hybrids, three 30-μl samples were taken from each capillary tube and mixed with 1.0 ml of 100 mM NaCl–50 mM sodium acetate (pH 6.0)–2 mM zinc chloride. Two of these samples were digested with 1,500 U of *Aspergillus oryzae* nuclease S_1 (510 U/μg) for 1 h at 37°C. After the addition of 100 μg of bovine serum albumin and 10 ml of 5% trichloroacetic acid to each sample, the precipitates were collected on Whatman GFC filters. The filters were dried and counted in a toluene-based scintillant. The average of the counts in the two samples digested with nuclease S_1 divided by the counts in the undigested sample yields the fraction of vaccinia virus DNA that had formed hybrids. A background ranging up to 3% was subtracted from all values. This background was not reduced by hydroxyapatite chroma-

tography to remove cross-linked DNA present naturally (1) or formed as a result of sonic treatment. The data were corrected for DNA-DNA reannealing (25), but in most cases this adjustment was small.

Synthesis of cDNA. [^{3}H]deoxyadenosine-labeled complementary DNA (cDNA) was synthesized in 300-μl reaction mixtures containing 50 mM Tris-hydrochloride (pH 8.3), 60 mM NaCl, 4 mM $MgCl_2$, 20 mM dithiothreitol, 0.6 mM each dCTP, dTTP, and dGTP, 0.1 mM [^{3}H]dATP (13 Ci/mmol), 100 μg of actinomycin D per ml, 20 μg of oligo(dT)$_{12-18}$ per ml, 100 to 200 μg of polyadenylylated RNA per ml, and 50 U of avian myeloblastosis virus reverse transcriptase (22 U/μg). Template RNA and oligo(dT) were first allowed to anneal for 15 min at 37°C, and after the addition of deoxynucleoside triphosphates and enzyme, the reaction mixture was incubated for 1 h at 37°C. Synthesis was stopped by the addition of 0.5% SDS–10 mM EDTA, and the mixture was adjusted to 0.3 N sodium hydroxide and boiled for 5 min. After neutralization with NaH_2PO_4, the ^{3}H-labeled cDNA was chromatographed on Sephadex G-75 in 5 mM ammonium acetate. The material in the void volume was precipitated with 0.1 M NaCl–75% ethanol after the addition of 100 μg of yeast RNA. The cDNA was collected from ethanol by centrifugation and then sedimented on a 5 to 20% sucrose gradient in 10 mM sodium acetate (pH 6.0)–0.1 M NaCl–0.1% SDS in a Beckman SW41 rotor at 35,000 rpm for 5 h at 17°C. cDNA sedimenting from 5 to 12S was precipitated with 75% ethanol after the addition of 100 μg of yeast RNA.

Analysis of data. For sequence complexity determinations in which the initial RNA concentration × time (R_0t) was varied as a function of time, final saturation values were obtained by averaging all data points in the plateau regions. For more detailed kinetic experiments in which R_0t was varied as a function of RNA concentration, least-squares best-fit curves given in equations 3 and 5 in the Appendix were calculated.

The program MLAB, designed by G. Knott of the National Institutes of Health, was used to find the best-fit curve by determining the number of components in a multiple exponential equation by increasing iteration until a constant sum of squares was obtained. This program was used to plot Fig. 4 through 8.

Source of materials. Poly(U)-Sepharose was purchased from Pharmacia Fine Chemicals or prepared with CNBr-activated Sepharose (Pharmacia) and poly(U) (P-L Biochemicals). Nuclease S_1 (*A. oryzae*) and oligo(dT)$_{12-18}$ were purchased from Miles Laboratories. Ribonucleoside and deoxyribonucleoside triphosphates were obtained from P-L Biochemicals. Cytosine arabinoside, cycloheximide, and protease K were obtained from Sigma Chemical Co., The Upjohn Co., and EM Laboratories, respectively. Avian myeloblastosis virus reverse transcriptase was a gift from J. W. Beard (Life Sciences, Inc.), and [^{3}H]thymidine and [^{3}H]dATP were purchased from the New England Nuclear Corp.

RESULTS

Difference in sequence complexity of polyadenylylated viral RNA synthesized at early and late times.

The extent of the large vaccinia virus genome transcribed at early (2 h) and late (7 h) times after infection of HeLa cells with vaccinia virus was investigated. Initial experiments were carried out using highly purified virus for infection. We confirmed that, after infection at 15 PFU/cell, viral DNA synthesis was maximal at 3 h and essentially completed at 7 h. Synthesis of characteristic [^{35}S]methionine-labeled late virus-specific polypeptides was detected at 3 to 4 h by electrophoresis in SDS-containing polyacrylamide gels (J. Cooper, personal communication). To assure that RNA extracted at 2 h was entirely early, i.e., synthesized before virus DNA replication, the cells were infected in the presence of cytosine arabinoside (40 μg/ml), an inhibitor of DNA synthesis. In the presence of this inhibitor, both total and cytoplasmic DNA syntheses were decreased by more than 99%. In addition, the polypeptide pattern from cells infected in the presence of cytosine arabinoside did not display any characteristic late virus-specific polypeptides even after incubation for several hours. Satisfied that polyadenylylated RNA obtained at 2 h postinfection in the presence of cytosine arabinoside (CAR RNA) can be classified as prereplicative, we began hybridization experiments. When an excess of CAR RNA was incubated for varying times with [^{3}H]thymidine-labeled vaccinia virus DNA, approximately 37% of the DNA became resistant to the action of nuclease S_1, an enzyme that digests single-stranded nucleic acids (Fig. 1 and Table 1). The RNA-DNA hybridization reaction was completed at a time when DNA-DNA hybridization in the absence of viral RNA was less than 5%. This value was not subtracted since DNA-DNA annealing must be even less in the presence of viral RNA. Approximately 40% of vaccinia virus DNA was also protected by polyadenylylated RNA (CYCLO RNA) obtained from cells at 4 h after infection in the presence of 100 μg of cycloheximide per ml (Fig. 1 and Table 1). Under the latter conditions, cycloheximide prevents protein synthesis needed for both virus uncoating and DNA replication.

Further experiments were carried out with cytoplasmic polyadenylylated RNA present at 7 h (LATE RNA) after vaccinia virus infection. LATE RNA protected approximately 41% of the labeled vaccinia virus DNA from nuclease S_1 digestion (Fig. 1 and Table 1). A combination of CAR RNA and LATE RNA protected 43% of the DNA, suggesting that the RNA sequences found "late" in infection with purified vaccinia virus include most or all of those found at early times.

The small difference in sequence complexity

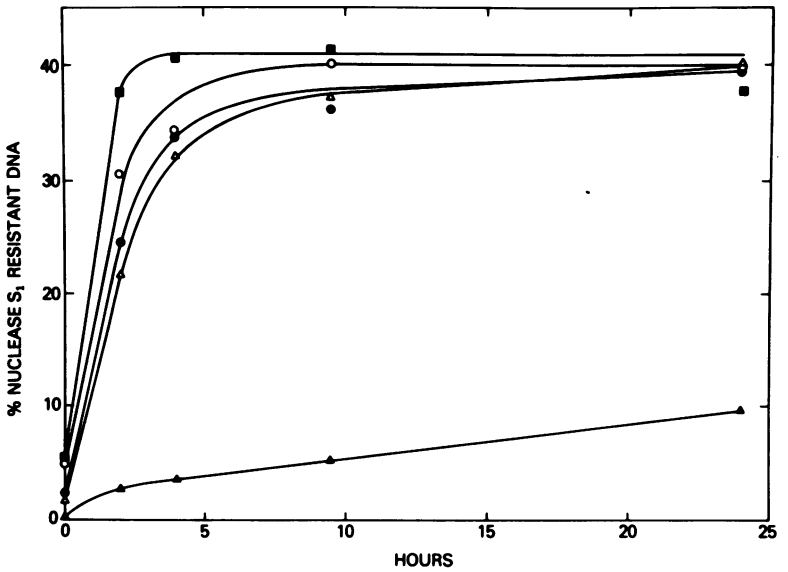

FIG. 1. *DNA-RNA hybridization with excess polyadenylylated RNA synthesized after infection with purified and sonically treated virus. Cytoplasmic polyadenylylated RNAs were obtained at 2 h after infection in the presence of cytosine arabinoside (CAR RNA), 7 h after infection without inhibitors (LATE RNA), and 4 h after infection with cycloheximide (CYCLO RNA) and hybridized to [^{3}H]thymidine-labeled vaccinia DNA in vast RNA excess at 68°C in 1.0 M NaCl-10 mM Tris-hydrochloride, pH 7.6. Samples were assayed at the indicated times for the formation of hybrids by digestion with nuclease S_1. The percentage of nuclease S_1-resistant DNA was calculated as described. Symbols: ●, 36 μg of CAR RNA per ml; △, 6 μg of LATE RNA per ml; ○, 34 μg of CYCLO RNA per ml; ■, 36 μg of CAR RNA per ml plus 6 μg of LATE RNA per ml; ▲, 40 μg of uninfected HeLa cell RNA per ml to show DNA-DNA hybridization.*

between CAR and LATE RNAs was surprising in view of obvious differences in the virus-specific polypeptides synthesized at early and late times. The possibility that a significant proportion of the RNAs are not translated early in infection appeared unlikely since the sequence complexities of total cytoplasmic polyadenylylated RNA and that fraction of the RNA obtained from $MgCl_2$-precipitated polyribosomes (38) were similar (Table 1). However, since hybridization was carried out in vast RNA excess, a very small amount of late mRNA synthesized at early times could account for the similarities in sequence complexities of CAR and LATE RNAs without the synthesis of detectable amounts of late proteins. A further possibility that such a small amount of putative late RNA sequences was made prematurely by defective particles formed during the purification of the inoculum virus was considered. That this hypothesis might be correct was suggested by repeating the previous experiments with unpurified virus inoculum.

In contrast to the previous results, cytoplasmic polyadenylylated RNA obtained at 2 h after infection with unpurified virus in the presence of cytosine arabinoside hybridized to only 26% of vaccinia DNA (Fig. 2 and Table 1). This value is approximately 60% of the total sequence com-

TABLE 1. *Percent nuclease S_1-resistant vaccinia virus DNA at mRNA saturation*[a]

RNA[b]	Nuclease S_1-resistant DNA (%)		
	Purified virus	Crude virus	Virus purified without sonic treatment
CAR	37 ± 3	26 ± 2	26
CAR polysomal	34		
CYCLO	40		28
LATE	41 ± 4	43	41
LATE polysomal	41		
CAR + LATE	43 ± 3		
In vitro	38 ± 2		42
In vitro + CAR	40		
In vitro + LATE	42		

[a] All values represent the average of duplicate nuclease S_1 digestions repeated several times after saturation was achieved. Standard deviations are included for experiments repeated with three to five separately prepared RNA samples.

[b] CAR RNA is polyadenylylated cytoplasmic RNA obtained at 2 h after infection in the presence of 40 μg of cytosine arabinoside per ml; CYCLO RNA is polyadenylylated cytoplasmic RNA obtained at 4 h after infection in the presence of 100 μg of cycloheximide per ml; LATE RNA is polyadenylylated cytoplasmic RNA obtained at 7 h after infection without inhibitors; in vitro RNA is polyadenylylated RNA made in vitro by vaccinia virus cores.

plexity of RNA present at late times (7 h) after infection with the same unpurified virus stock (Fig. 2 and Table 1).

In an attempt to determine which steps during virus purification led to the production of particles with defective transcriptional control, sonic treatment was omitted and trypsinization was used to disperse virus particles. The particle-to-PFU ratio of virus purified by either procedure was approximately 50 (see methods). Cytoplasmic polyadenylylated RNA obtained at 2 h after infection with purified but unsonicated virus in the presence of cytosine arabinoside was hybridized to vaccinia virus DNA. This RNA also hybridized to only 26% of the viral DNA (Fig. 2 and Table 1), suggesting that sonic treatment rather than sucrose gradient sedimentation is responsible for producing damaged particles that exhibit loss of transcriptional control.

To summarize this segment of the work, we found that when either unpurified vaccinia virus or virus purified without sonic treatment steps was used for infection, polyadenylylated RNA sequences corresponding to approximately 26% of the total DNA are present early and that sequences corresponding to approximately 42% of the DNA are present late. This degree of sequence complexity would correspond to 94 early and 152 late mRNA's of 1,000-nucleotide length. As discussed later, both the sequence complexities of LATE RNA and the addition experiments with CAR and LATE RNAs may be underestimates resulting from the presence of self complementary RNA sequences.

Sequence complexity of RNA synthesized in vitro by vaccinia virus cores. The sequence complexity of RNA synthesized in vitro by enzymes present within vaccinia virus cores was determined. When virus purified by procedures that included sonic treatment was used, excess polyadenylylated RNA was found to protect 38% of vaccinia virus DNA from digestion by nuclease S_1 (Fig. 3 and Table 1). The addition of either CAR RNA or LATE RNA, obtained from cells infected with purified (sonically treated) virus, to in vitro synthesized RNA resulted in little, if any, additional protection of vaccinia virus DNA (Fig. 3 and Table 1), suggesting that in vitro transcription produces only those sequences found in vaccinia virus-infected cells. No significant difference was found when virus purified without sonic treatment was used for in vitro RNA synthesis (Fig. 2 and Table 1), implying that in vitro conditions permit some loss of transcriptional control.

Abundance classes of RNAs determined by hybridization to virion DNA. mRNA's present in animal cells (for a review, see refer-

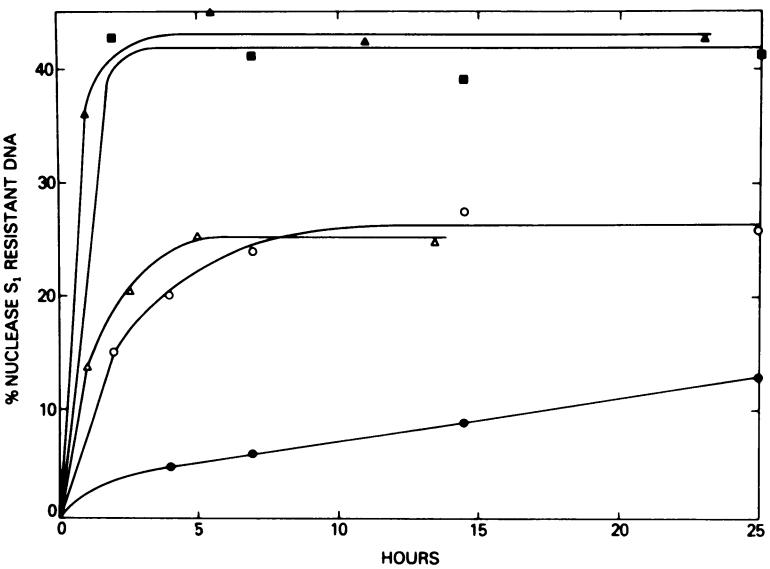

FIG. 2. *DNA-RNA hybridization with excess polyadenylylated RNA synthesized after infection with either unpurified virus or virus purified without sonic treatment. Hybridization of [^{3}H]thymidine-labeled DNA with cytoplasmic polyadenylylated RNAs was assayed by resistance to single-strand-specific nuclease S_1. RNAs were obtained from cells at 2 h after infection with 15 PFU of crude virus per cell in the presence of cytosine arabinoside (○, 124 μg/ml) or at 7 h after infection without inhibitors (▲, 150 μg/ml). Virus purified without sonic treatment was used for infection of HeLa cells in the presence of cytosine arabinoside (△, 203 μg/ml) and for in vitro RNA synthesis (■, 184 μg/ml) as described in the next legend. Uninfected HeLa cell RNA (●, 40 μg/ml).*

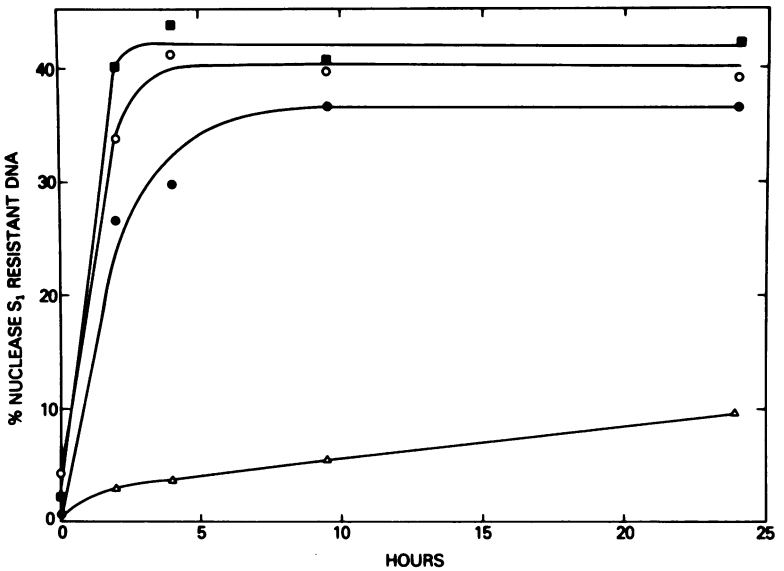

FIG. 3. *DNA-RNA hybridization to excess polyadenylylated RNA synthesized in vitro. Poly(A)-containing RNA synthesized in vitro using purified (sonically treated) vaccinia virus (●, 13 μg/ml) was hybridized in excess to [^{3}H]thymidine-labeled vaccinia virus DNA. Samples were assayed for the formation of DNA-RNA hybrids as described in the text. Polyadenylylated RNAs synthesized in vivo after infection with purified virus were combined with in vitro-synthesized RNA: ○, CAR RNA (36 μg/ml) plus in vitro RNA (13 μg/ml); ■, LATE RNA (7 μg/ml) plus in vitro RNA (13 μg/ml). A control reaction contains uninfected HeLa cell polyadenylylated RNA (△, 40 μg/ml).*

ence 27), yeast (18), and virus-infected cells (8–10) have been shown by hybridization kinetics to exist in discrete classes varying in abundance by as much as 1,000-fold. Three approaches have been used to define abundance classes present in heterogeneous RNA populations: (i) hybridization of excess mRNA to radioactively labeled viral (9, 10) or single-copy eucaryotic (11, 12, 18) DNA; (ii) hybridization of excess mRNA to its radioactively labeled cDNA (2, 14, 17–20, 26, 41, 46, 47); and (iii) hybridization of mRNA to excess, radioactively labeled, purified DNA fragments (8). We have looked for abundance classes in vaccinia virus RNA synthesized in vivo and in vitro by the first two of these methods.

Varying concentrations of cytoplasmic polyadenylylated CAR RNA obtained at 2 h after infection with unpurified vaccinia virus were hybridized for 16 h to [^{3}H]thymidine-labeled vaccinia virus DNA, and the fraction of DNA forming hybrids was determined as before by resistance to nuclease S_1 digestion. When the fraction of hybridized DNA was plotted versus log R_0t, the data approximated a pseudo first-order exponential curve (Fig. 4), suggesting that a single abundance class of RNA drives the hybridization reaction. Values of α_n and β_n (see Appendix) derived from a least-squares best fit to two independent sets of data are presented in Table 2. As explained in the Appendix, the value of 0.277 for α_n is the fraction of vaccinia virus DNA complementary to CAR RNA and is similar to the value in Table 1 obtained from saturation experiments. The R_0t at which the hybridization reaction is half completed ($R_0t_{1/2}$) can be used to determine the proportion of the CAR RNA that appears to be driving the reaction (11). To do this, it is necessary to derive the expected $R_0t_{1/2}$ from both the measured complexity of CAR RNA and the $R_0t_{1/2}$ for a pure RNA standard of known complexity. The latter was provided by hybridization of sheep α- and β-globin mRNA's to their homologous cDNA (see Fig. 7). The $R_0t_{1/2}$ obtained for the two globin RNA chains, each of which contains approximately 590 transcribed nucleotides and 50 nontranscribed adenylic acid residues (6, 28), was 1.43×10^{-2} mol·s/liter, which agrees with published values for similar size probes (2) when corrected for the higher salt concentration used in our hybridization experiments (4). From this value, the expected $R_0t_{1/2}$ for a single class of CAR RNA complementary to 26% of the vaccinia virus genome would be 1.05. Dividing the observed $R_0t_{1/2}$ of 0.884 by the expected $R_0t_{1/2}$ leads to the conclusion that 84% of the RNA drives the hybridization reaction shown in Fig. 4. This result suggests that the majority of the total cytoplasmic polyadenylylated RNA se-

quences present in HeLa cells at 2 h after infection are viral. We wish to emphasize that high abundance classes of RNA hybridizing to a very small percentage of the vaccinia virus genome are difficult to resolve by this method.

Notable differences were found when similar experiments were carried out with LATE RNA. From a comparison of the observed and calculated $R_0t_{1/2}$, only 16% of the RNA appeared to be driving the hybridization reaction, whereas, as will be discussed later, 82 to 92% of the total polyadenylylated cytoplasmic RNA may be virus specific at this time. A result such as this can be explained if viral RNA species exist in multiple abundance classes. Since hybridization of the viral RNA species present at low concentrations will be rate limiting, only a fraction of the total viral RNA will appear to be driving the reaction. Further support for multiple abundance classes was obtained from analysis of the hybridization curve, which extended over more than 2 log-decades and was best fit to a two-component exponential equation (Fig. 5). The presence of two classes of RNA hybridizing to 22.2 and 19.5% of the DNA and differing 11-fold in abundance was indicated by the least-squares

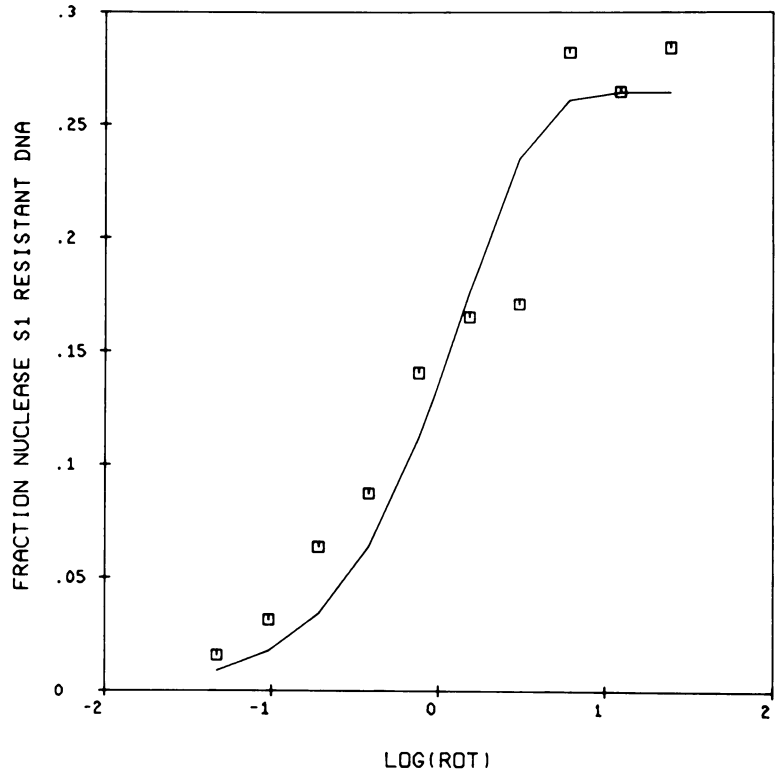

FIG. 4. *Hybridization of CAR RNA with vaccinia virus DNA. Polyadenylylated RNA obtained at 2 h after infection with crude vaccinia virus in the presence of cytosine arabinoside was hybridized to [^{3}H]thymidine-labeled vaccinia virus DNA. The formation of hybrids was assayed by resistance to nuclease S_1. The solid line is the least-squares best fit to equation 3 of the Appendix (n = 1).*

TABLE 2. *RNA abundance classes determined by hybridization of polyadenylylated RNA to vaccinia virus DNA*

RNA	No. of RNA abundance classes	α^a	β^a (liter/mol·s)	Relative abundance
CAR	1	0.277 ± 0.013	0.713 ± 0.149	1
LATE	2	0.222 ± 0.029	5.18 ± 1.49	11.4
		0.195 ± 0.027	0.455 ± 0.105	1
In vitro	2	0.201 ± 0.012	13.2 ± 2.6	43.4
		0.167 ± 0.013	0.303 ± 0.059	1

[a] See Appendix for sum of exponentials equation to which data were fit and for definitions of α and β.

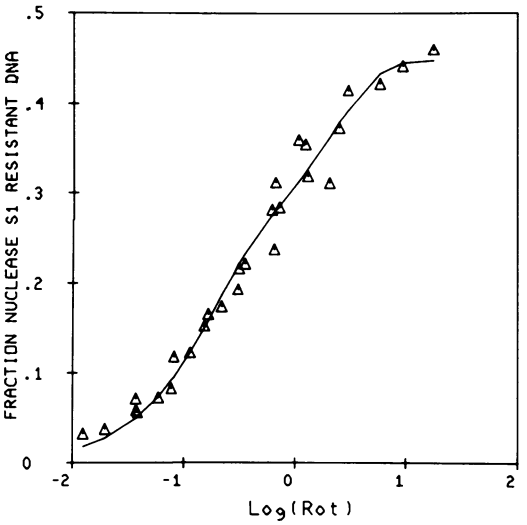

FIG. 5. *Hybridization of LATE RNA with vaccinia virus DNA. Polyadenylylated RNA obtained at 7 h after infection with vaccinia virus was hybridized to [^{3}H]thymidine-labeled vaccinia DNA. The fraction of nuclease S_1-resistant DNA was determined as described in the text. The curve drawn is the least-squares best fit to equation 3 of the Appendix (n = 2).*

best fit to three independent sets of data (Table 2). The saturation value a_n of 0.417 (Table 2) was similar to that determined in RNA saturation experiments (Table 1). Again, we emphasize that this method is not well suited for the resolution of abundance classes of RNA hybridizing to a very small percentage of the genome.

Analysis of polyadenylylated RNA synthesized in vitro by vaccinia virus particles is simplified by the absence of any host sequences. Nevertheless, from the observed $R_0t_{1/2}$/expected $R_0t_{1/2}$, the hybridization reaction appears to be driven by only 15% of the RNA. Since all of the RNA made in vitro is viral, this result is also best explained by the existence of abundance classes of viral RNA. DNA-RNA hybridization extended over 3 log-decades, and the curve was fit best by a two-component exponential equation (Fig. 6). Classes of RNA representing 20.1 and 16.7% of viral DNA sequences and differing 43-fold in abundance were calculated by using two independent sets of data (Table 2). It is possible that the class of in vitro RNA present in low abundance contains aberrant transcripts of late sequences.

Abundance classes of RNA determined by hybridization to cDNA. DNA complementary to polyadenylylated RNA (cDNA) can be synthesized by using the enzyme reverse transcriptase. Evidence has been obtained, by mixing globin RNA with total cell mRNA, that cDNA prepared from a population of RNAs is an accurate complement of that population (17). The unique advantage of a cDNA probe is that a large proportion of it will be complementary to abundant RNAs, even if the latter have a very low sequence complexity. Use of cDNA prepared to in vivo polyadenylylated mRNA is complicated, however, by the admixture of viral and host sequences.

The fraction of a [^{3}H]deoxyadenosine-labeled cDNA probe, prepared using polyadenylylated RNA obtained from cells at 2 h after infection with unpurified vaccinia virus in the presence of cytosine arabinoside, that is virus specific was estimated by hybridization to unlabeled vaccinia virus DNA. At least 52% of the cDNA probe was hybridized (data not shown). This value, although lower than the 84% predicted from the percentage of CAR RNA driving the hybridization to virus DNA in the previous section, agreed with the reciprocal experiment of hybridizing polyadenylylated RNA from uninfected cells to the CAR cDNA probe; approximately 41% of the cDNA probe hybridized to HeLa cell mRNA (Fig. 7).

Kinetic data obtained by hybridization of CAR RNA from infected cells to its own cDNA probe (Fig. 7) was fit to a curve defining the sum of multiple pseudo first-order equations (see Appendix). Computer analysis of the data indicated that three abundance classes of polyadenylylated RNA are present at 2 h after infection (Fig. 7). The $R_0t_{1/2}$ observed for each RNA class, as well as the theoretical $R_0t_{1/2}$ that would result from the hybridization of the RNA of a single abundance class with its cDNA only, can be calculated from n, a_n, and k_n, which define the least-squares best-fit curve. Two high abundance classes were found differing 13-fold in relative abundance and together comprising approximately 64% of the total RNA. The nucleotide complexity of the RNA sequences in each abundance class can be determined from $R_0t_{1/2}$ (if pure) values (Table 3 and Appendix) by using a well-defined standard. As before, hybridization of sheep α- and β-globin mRNA's to their own probes were used (Fig. 7). Assuming that the average cytoplasmic polyadenylylated RNA in vaccinia virus-infected cells contains 1,100 nucleotides, 100 of which are nontranscribed adenylic acid residues at the 3′ end, we calculated that there are approximately 6.7 and 141 RNA species in the two abundant classes that comprise 64% of the total RNA. These classes must contain the majority of the 94 early virus-specific sequences calculated from the complexity data and may also contain some abundant cell mRNA's. It is necessary to point out that the

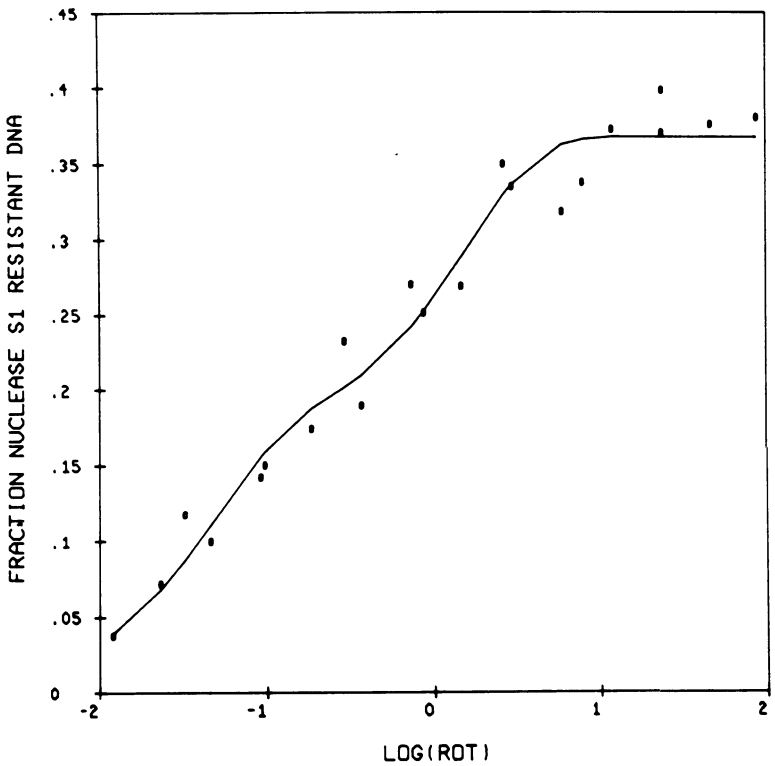

FIG. 6. *Hybridization of in vitro RNA with vaccinia virus DNA. Varying concentrations of polyadenylylated RNA synthesized in vitro were hybridized to [^{3}H]thymidine-labeled vaccinia virus DNA, and the fraction of DNA forming hybrids was determined by its resistance to nuclease S_1 digestion. The curve is the least-squares best fit to the data for the sum of multiple exponentials as given in equation 3 of the Appendix (n = 2).*

number of RNA molecules calculated from the hybridization data is based on the assumption that each RNA contains 1,000 nucleotides (1 kilobase) of unique sequence information. This number should be considered only an approximation of the true number of different viral RNA molecules contained in a given abundance class. The nucleoside complexity of the RNA class of highest abundance represents less than 2% of that in the large vaccinia virus genome and was not detected by hybridization to single-copy virion DNA in the previous section. We have not attempted to carefully define the least abundant class of RNA, which must contain the majority of cell mRNA's.

Similar experiments were carried out with [^{3}H]deoxyadenosine-labeled cDNA synthesized by using polyadenylylated RNA obtained at 7 h after infection with unpurified virus as template. Approximately 82% of this cDNA probe hybridized to vaccinia virus DNA, indicating that the LATE RNA from which it was prepared was predominantly virus specific. This value is a minimal one since sufficient DNA to saturate the most abundant RNAs could not be added. Up to 92% of the cDNA could be virus specific since only 8% of the cDNA hybridized to polyadenylylated cytoplasmic RNA from uninfected HeLa cells (Fig. 8). Data obtained from hybridization of the LATE RNA with its own cDNA and a three-component, least-squares best-fit curve to these data are shown in Fig. 8. Data obtained from four different experiments indicated that the three classes of polyadenylylated RNA hybridize to 34.0, 34.8, and 20.2% of the cDNA probe, respectively (Table 3). Clearly, the two abundant classes of RNA must be composed predominantly of viral sequences since they hybridize to 69% of the cDNA. Using the hybridization of sheep α- and β-globin mRNA's to their own cDNA as a standard, we calculated that the equivalent of 0.72, 8.3, and 208 RNA sequences of 1,000-nucleotide lengths are present in the three classes (Table 3). The mRNA's of the two high abundance classes are present approximately 500 and 40 times more frequently than those of the least abundant class. Together the RNA sequences present in the high abundance classes would hybridize to approximately 2% of the vaccinia virus DNA and were not detected

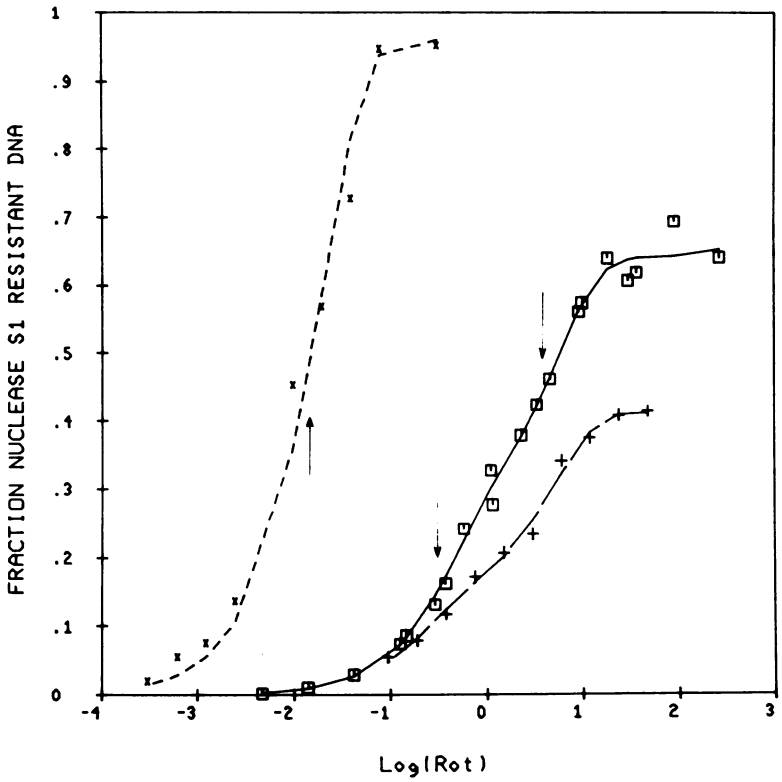

FIG. 7. *Hybridization of CAR RNA to cDNA. Polyadenylylated RNA obtained at 2 h after infection with crude virus in the presence of cytosine arabinoside* (□) *was hybridized to [^{3}H]deoxyadenosine-labeled cDNA that was synthesized using CAR RNA as template. The fraction of nuclease S_1-resistant DNA was determined. The curve (solid line) is the least-squares best fit to the data for equation 5 of the Appendix (n = 3). The downward arrows* (↓) *indicate the $R_0t_{1/2}$(observed) for the two RNA classes of high relative abundance. Uninfected HeLa cell* (+) *polyadenylylated RNA was hybridized to heterologous CAR cDNA, and the dashed line is a least-squares best fit to equation 3 of the Appendix (n = 2). A saturation value of 0.412 was reached. A least-squares best-fit curve to equation 3 of the Appendix was determined for the hybridization of sheep α- and β-globin mRNA's to their homologous cDNA* (×). *The upward arrow* (↑) *indicates the $R_0t_{1/2}$(observed) value of 0.0143 mol·s/liter.*

by hybridization to single-copy virion DNA in the previous section. The presence of such abundant RNA species accounts for the relatively low proportion of the viral RNA driving the latter hybridization reaction. Most of the 152 total viral RNA sequences are present in the least abundant class of RNA.

To determine the proportion of the viral RNA present at 7 h that contains specific early sequences, CAR RNA was hybridized to the labeled cDNA probe prepared from LATE RNA. CAR RNA hybridized to approximately 34% of this cDNA (Fig. 8), a number greater than that found in the RNA class of lowest abundance (20%). It is possible, therefore, that synthesis of one or more of the abundant late sequences begins at an early time. Uninfected HeLa cell RNA, on the other hand, is complementary to only 8.2% of the cDNA probe and has a high $R_0t_{1/2}$ value, making it unlikely that any host sequences are in high abundance at 7 h after infection (Fig. 8).

Our finding that only a low proportion of the viral RNA synthesized in vitro by vaccinia virus particles apparently drives hybridization to virion DNA led us to consider the presence of a low complexity, high abundance class of in vitro RNA. To examine this further, [^{3}H]deoxyadenosine-labeled cDNA was prepared, using polyadenylylated in vitro RNA as a template. The kinetics of hybridization of unlabeled in vitro RNA to its own cDNA probe suggested the presence of two classes of RNA differing approximately 150-fold in relative abundance (Fig. 9 and Table 3). Using the $R_0t_{1/2}$ (observed) value of 0.0143 mol·s/liter obtained for the hybridization of sheep α- and β-globin RNAs to their homologous cDNA probes as a standard, we calculated that the equivalents of 1.6 and 77 RNAs of 1-kilobase complexity are present in

TABLE 3. *RNA abundance classes determined by hybridization of polyadenylylated RNA to cDNA*[a]

RNA	No. of RNA classes	α	k (liter/mol·s)	$R_0t_{1/2}$ (observed) (mol·s/liter)[b]	$R_0t_{1/2}$ (if pure) (mol·s/liter)[c]	No. of RNA sequences[d,e]	Relative abundance
Sheep α- and β-globin mRNA's	1	0.960	50.4	0.0143	0.0143		
CAR	3	0.250	9.43	0.293	0.082	6.7 (4.9–10.6)	11,500
		0.388	0.447	3.99	1.72	141 (122–166)	850
		0.261	0.000784	3,380	982	80,300 (26,300–>10^6)	1
LATE	3	0.340	87.6	0.023	0.00880	0.72 (0.46–1.58)	496
		0.348	7.55	0.264	0.102	8.3 (6.2–12.5)	43
		0.202	0.303	11.4	2.55	208 (161–292)	1
In vitro	2	0.694	38.6	0.0259	0.200	1.6 (1.5–1.8)	157
		0.208	0.820	4.056	0.939	77 (60–107)	1

[a] Data obtained from least-squares best-fit curves to data obtained from two (CAR and in vitro) or four (LATE) separate RNA preparations. See Appendix for equation and definitions of α and k.

[b] $R_0t_{1/2}(\text{observed}) = \ln 2/\alpha k$

[c] $R_0t_{1/2}(\text{if pure}) = \ln 2/0.9k$

[d] Number of sequences = $[R_0t_{1/2}(\text{if pure})/R_0t_{1/2}\ \text{globin}] \times 1.17$, which corrects for 7.8% poly(A) content of globin mRNA and standardizes for polyadenylylated RNAs of 1,100 nucleotides in length, including a 100-nucleotide poly(A) tract (see text).

[e] Range obtained from standard error in $R_0t_{1/2}$(if pure) value.

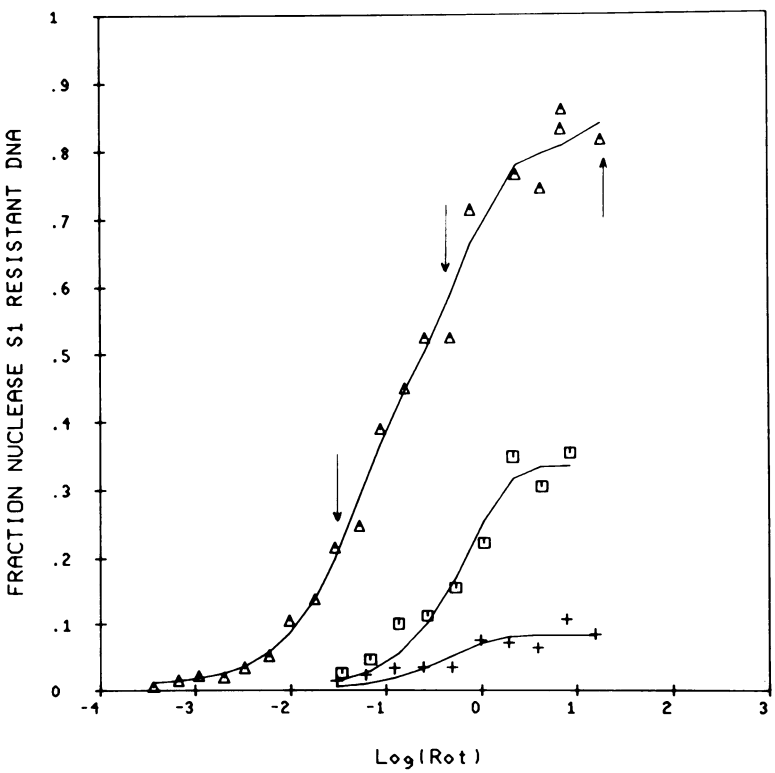

FIG. 8. *Hybridization of LATE RNA to cDNA. Polyadenylylated RNA obtained at 7 h after infection* (△) *was hybridized with [^{3}H]deoxyadenosine-labeled cDNA that was synthesized using LATE RNA as template. The least-squares best fit to the data for the curve given by equation 5 of the Appendix (n = 3) is shown. The arrows indicate the $R_0t_{1/2}$(observed) for the two high abundance* (↓) *and the low abundance* (↑) *RNA classes. CAR RNA* (□) *and uninfected HeLa cell* (+) *polyadenylylated RNA, when hybridized to heterologous LATE cDNA and fit to equation 3 of the Appendix, yielded saturation values of 0.339 and 0.082, respectively.*

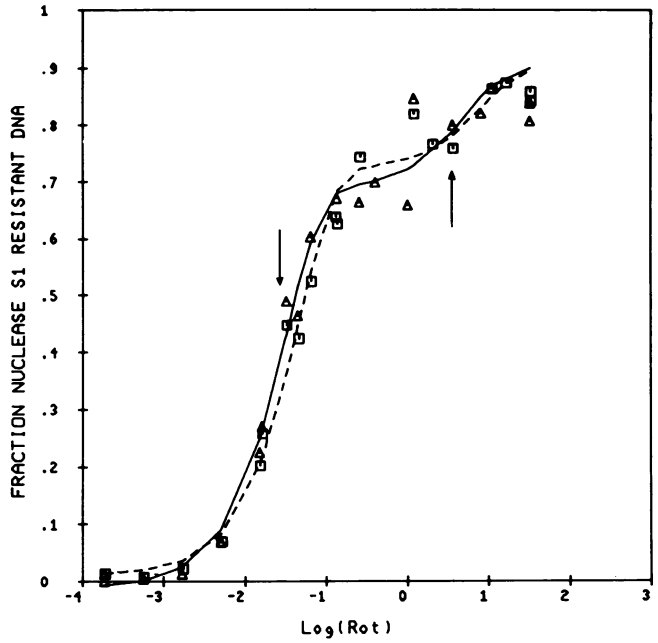

FIG. 9. *Hybridization of in vitro-synthesized RNA to cDNA. Polyadenylylated RNA synthesized in vitro and obtained either by cetyltrimethylammonium bromide precipitation alone* (□) *or by additional pelleting in CsCl* (△) *was hybridized with homologous [^{3}H]deoxyadenosine-labeled cDNA, and the formation of RNA-cDNA hybrids was assayed by resistance to nuclease S_1. The curves represent a least-squares best fit to equation 5 of the Appendix (n = 2) for each set of data. The arrows indicate the $R_0t_{1/2}$(observed) for the high* (↓) *and low* (↑) *RNA abundance classes calculated using both sets of data.*

the high and low abundance classes, respectively. It appears likely that some of the abundant RNAs synthesized in vitro and at early times in vivo are similar since a class of in vitro RNA was found to hybridize with an $R_0t_{1/2}$ (observed) of 0.0408 mol·s/liter to approximately 26% of the labeled CAR cDNA probe (data not shown).

DISCUSSION

Poxviruses have the largest genomes of any group of animal viruses. The best estimate of the molecular weight of vaccinia virus DNA appears to be 123×10^6 (13), which agrees with its sequence complexity (16). Since vaccinia virus mRNA has an average length of 1,100 nucleotides, as determined by end group analysis (3), of which approximately 100 form nontranscribed 3'-polyadenylic acid [poly(A)] (35), there is a potential for 362 nonoverlapping mRNA species. If the assumption is made that only noncomplementary regions of the DNA are transcribed, this potential number is reduced to 181. This is a minimal number since there is, in fact, evidence for vaccinia virus RNA that is complementary (5; Boone, unpublished data). To determine the sequence complexity of vaccinia virus RNA, we employed liquid hybridization of excess polyadenylylated cytoplasmic RNA to a labeled, denatured, vaccinia virus DNA probe. Under these conditions, DNA-DNA annealing is minimal. The values obtained by this method may be underestimates, however, if RNA-RNA hybridization effectively competes with RNA-DNA hybridization. Our unpublished experiments indicate that significant amounts of late (7 h) polyadenylylated virus-specific RNA species form hybrids with themselves and with early RNA species, but the extent to which this competition lowers the final amounts of DNA hybridized cannot be accurately determined by our present methods. With this reservation in mind, we have determined that approximately 42% of the viral DNA, equivalent to 152 RNA species, can be hybridized to an excess of polyadenylylated RNA obtained from the cytoplasm or polyribosomes of cells at 7 h after infection.

Two types of transcriptional controls were identified, one of which can be discerned by simple complexity measurements. Thus, early viral mRNA made at 2 h after infection in the presence of an inhibitor of DNA synthesis hybridizes to 26% of the viral DNA corresponding to 94 RNA species of 1,000-nucleotide length. The early RNA sequences may simply represent a subset (60%) of the 152 late RNA species since

additional DNA is not protected by a mixture of early and late RNA. Interpretation of these results, however, is complicated by some annealing of early RNA with late RNA (Boone, unpublished data). Nevertheless, these values agree with the competition hybridization experiments of Oda and Joklik (36), which indicated that hybridization of one-half to two-thirds of the late vaccinia virus-specific RNA can be competed by early RNA. Our results are also quite similar to the recent report by Paoletti and Grady (39) that early and late RNAs hybridize to 25 and 52% of the vaccinia virus DNA. The variation between the values obtained for late RNA may be due to differences in the hybridization conditions or method of analysis of hybrids. It is not due to the use of total RNA by Paoletti and Grady (39), since we also obtained approximately 42% hybridization when total RNA was used in place of polyadenylylated RNA. Similar experiments carried out with rabbit poxvirus RNA by Kaverin and co-workers (25) indicated that early and late RNAs are complementary to 17 to 20% and 40 to 42% of the DNA.

A second type of transcriptional control was discerned by RNA abundance measurements. Two approaches, hybridization of unlabeled polyadenylylated RNA to vaccinia virus DNA and hybridization to its own cDNA prepared with reverse transcriptase, were used. The first approach is most direct, since only virus-specific RNAs are measured, and has previously been used with herpesvirus (9, 10). However, it is difficult to detect abundant RNAs of very low sequence complexity by this procedure. The second approach, used extensively to study eucaryotic mRNA (for a review, see reference 27), is ideally suited to define high abundance-low complexity RNAs but measures low abundance RNAs less accurately than the first. It should also be realized that the classes of RNA defined by these methods differ in average abundance and that almost certainly each is composed of subclasses. Unambiguous evidence for multiple abundance classes was obtained with late mRNA. Data obtained by hybridization of late mRNA with virus DNA was computer fit to a two-component exponential equation. Two classes of virus-specific RNA differing 11-fold in abundance were indicated. Data obtained by hybridization to cDNA, however, was best fit to a three-component exponential equation. The two most abundant classes together represented 69% of the RNA but contained less than 5% of the sequence complexity. Since this RNA would hybridize to only 2% of the vaccinia virus DNA, it is detected most easily by hybridization to cDNA. That these are viral sequences, however, is evident from the fact that 82 to 92% of the cDNA probe was shown to be virus specific. Assuming that the abundant sequences are all present in separate RNAs of 1,000-nucleotide length, approximately nine RNA molecules are present 40 to 500 times more frequently than the bulk of the virus-specific RNA. It is reasonable to speculate that these mRNA's code for the major virus structural proteins. The remaining 140 or more virus-specific RNA sequences that comprise only 20% of the total polyadenylylated RNAs present late in infection are divided into two classes, differing 11-fold in abundance, by analysis of RNA hybridization to virion DNA.

In contrast to the results obtained with late RNA, hybridization of polyadenylylated early RNA to virion DNA provided data that were best fit to a pseudo first-order exponential equation, suggesting a single class. Hybridization to homologous cDNA indicated three classes; however, only about 55% of the cDNA probe was virus specific, and the lowest abundance class representing 26% of the RNA undoubtedly consisted of host sequences. The 148 sequences calculated for the two abundant RNA classes included the majority of the 94 virus-specific sequences. The highest abundance class, corresponding to only seven sequences in 13-fold molar excess over the majority of virus-specific RNAs, probably also contains virus-specific sequences. However, definitive evidence to support this was not obtained since this class represented only 25% of the total RNA and its complexity was too low to analyze by hybridization to virion DNA.

The basis for restricted transcription of the vaccinia virus genome at early times and for the abundance classes that are particularly evident at late times is unkown. Since only steady-state concentrations of virus-specific RNA have been measured in this study, variations in RNA abundance could result from variations in the rate of synthesis or degradation. The marked decreases in the stability of vaccinia virus mRNA that occur after DNA replication (36, 42) could be involved.

The usefulness of the cDNA probes was not limited to providing information regarding abundance classes of early and late mRNA. The finding that cDNA probes prepared with total polyadenylylated cytoplasmic RNA at 2 and 7 h after infection were more than 52 and 82% virus specific, respectively, was surprising. Provided that cell and viral mRNA's are used equally well as templates by reverse transcriptase, this result indicates either that viral RNA is made at a rate so prodigious that it rapidly exceeds the concentration of cell mRNA or that degradation of cell

mRNA or its poly(A) tail is enhanced by infection. In either case, the high ratio of viral to cellular mRNA is likely to be an important factor in the inhibition of host protein synthesis by vaccinia virus (29). The cDNA probe to late RNA was also used to determine the proportion of early sequences in late RNA. Approximately 34% of the late cDNA hybridized to CAR RNA, indicating that early sequences are still present in substantial amounts at 7 h after infection. This result is consistent with the continued synthesis of some early viral proteins throughout the growth cycle (30).

One unanticipated aspect of this study involved the loss of transcriptional control that was encountered in vivo and in vitro. In initial experiments carried out using highly purified virus for infections, excess early and late RNAs were found to hybridize to similar proportions of the genome. Premature synthesis of late sequences was avoided when unpurified virus was used for infection or when sonic treatment steps were omitted and the virus preparations were dispersed by trypsin treatment. No significant difference was found in the particle-to-PFU ratios of purified virus prepared by the two methods. Moreover, no differences were found in the virus-specific polypeptides synthesized before DNA replication after infection with unpurified or either type of purified virus. We suggest, therefore, that only a very small percentage of the CAR RNA synthesized after infection with purified (sonically treated) virus corresponds to late sequences and that this was revealed only because RNA excess hybridization was performed. A small percentage of particles are probably damaged by sonic treatment and lose constraints to late RNA synthesis. An alternative possibility not yet excluded, however, is that the trypsin treatment eliminates incomplete or damaged virus particles that would transcribe late sequences.

A second situation in which transcriptional control is lost occurs when purified virus is used to synthesize mRNA in vitro. We expected RNA synthesized in vitro by vaccinia virus particles to correspond to early in vivo RNA and consequently to hybridize to a maximum of 26% of the DNA. Instead, we found that with preparations of purified virus that were either sonically treated or not, from 38 to 42% of the DNA was transcribed. The extra sequences appeared to be identical to in vivo late RNA sequences since a mixture of in vitro RNA and LATE RNA still hybridized to 42% of the DNA. These results, although similar to those of Paoletti and Grady (39) and DeFilippes (personal communication), are different from the earlier values obtained by Kates and Beeson (22) and Nevins and Joklik (35). The latter groups estimated that labeled in vitro RNA hybridized to 7% (22) and 25% (35) of vaccinia virus DNA immobilized to filters. Although the lower values obtained in the earlier studies may signify that more restricted transcription occurred under the in vitro conditions used by these investigators, it seems likely that only a portion of the RNA species was measured by hybridization to DNA immobilized to filters because of wide differences in RNA abundance. Our analysis of the hybridization of in vitro RNA to its homologous cDNA indicated that a small proportion of the vaccinia genome is represented by the majority of the RNA synthesized in vitro and that these sequences are present 150-fold more often than the RNAs composing the bulk sequence complexity. Hybridization of in vitro RNA to a cDNA probe prepared with CAR RNA template suggested that some of the abundant in vitro RNAs are also abundant in vivo at early times. Independent support for this was obtained by the finding of similar polyacrylamide gel patterns of [^{35}S]methionine-labeled polypeptides synthesized in cell-free systems directed by in vitro and early in vivo RNAs (J. Cooper, personal communication).

The demonstration of discrete early and late vaccinia virus-specific polypeptides (for a review, see reference 30) is in general agreement with temporal changes in transcriptional complexity. Up to 80 virus-specific polypeptides have been detected by one-dimensional gel electrophoresis (compared with 152 potential mRNA's), 30 appearing before and 50 appearing after the onset of virus DNA synthesis (40). Additional methods such as SDS-hydroxylapatite chromatography (31) or two-dimensional gel electrophoresis (37) may be needed to resolve additional virus-specific polypeptides. Large differences observed in the relative amounts of viral polypeptides could be explained by differences in RNA abundance although variations in translational efficiencies of mRNA's may also be a factor. An important project for the future is to correlate transcriptional and translational products of individual vaccinia virus genes.

APPENDIX

A pseudo first-order RNA-driven reaction can be expressed by the equation:

$$D_t/D_0 = e^{-kR_0t} \quad (1)$$

where D_t is the concentration of DNA that remains single stranded at time t, D_0 is the initial concentration of DNA, R_0 is the initial concentration of RNA, and k is the rate constant that is equal to $\ln 2/R_0t_{1/2}$ (2). When multiple classes of RNA exist, the equation for the sum of multiple exponentials is used (9):

$$D_t/D_0 = 1 - (\alpha_1 + \ldots \alpha_n) + \alpha_1 e^{-k_1 R_0 t} + \ldots \alpha_n e^{-k_n R_0 t} \quad (2)$$

where α_n, R_n, and k_n are, respectively, the fraction of DNA complementary to, the RNA concentration of, and the rate constant for each RNA class n. Assuming that the base compositions of the various RNA species do not vary appreciably, the rate constant k_n is approximately equal for all classes and can be expressed as a constant K (9). Equation 2 therefore becomes:

$$D_t/D_0 = 1 - \sum \alpha_n + \sum \alpha_n e^{-\beta_n R_0 t} \quad (3)$$

where $\beta_n = KR_n/R_0$. Ratios of β_n give the relative abundance difference between two classes. When cDNA synthesized from an RNA sample is used as the probe instead of DNA, $R_n = \alpha_n R_0$ for every RNA class, and equation 2 becomes:

$$cD_t/cD_0 = 1 - \sum \alpha_n + \sum \alpha_n e^{-k_n \alpha_n R_0 t} \quad (4)$$

Since the probe is complementary to all of the sequences present in the input RNA (17), $\sum \alpha_n$ should equal 1. However, 0.90 was the maximum fraction of cDNA that could be protected from digestion by nuclease S_1 and, therefore, a curve with the equation:

$$cD_t/cD_0 = 0.1 + \sum \alpha_n e^{-k_n \alpha_n R_0 t} \quad (5)$$

was used in a least-squares best fit to the data. The $R_0 t_{1/2}$, which is observed for each class n, is equal to $\ln 2/k_n \alpha_n$, and the $R_0 t_{1/2}$ for an RNA class n, assuming it were the only class present, is given by the expression $R_0 t_{1/2}$ (if pure) $= \ln 2/0.9 k_n$ since $\alpha_n = 0.9$.

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